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Regulation and Localization of Key Enzymes during the Induction of Kranz-Less, C_4-Type Photosynthesis in *Hydrilla verticillata*[1]

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Kranz-less, C_4-type photosynthesis was induced in the submersed monocot *Hydrilla verticillata* (L.f.) Royle. During a 12-d induction period the CO_2 compensation point and O_2 inhibition of photosynthesis declined linearly. Phospho*enol*pyruvate carboxylase (PEPC) activity increased 16-fold, with the major increase occurring within 3 d. Asparagine and alanine aminotransferases were also induced rapidly. Pyruvate orthophosphate dikinase (PPDK) and NADP-malic enzyme (ME) activities increased 10-fold but slowly over 15 d. Total ribulose-1,5-bisphosphate carboxylase/oxygenase activity did not increase, and its activation declined from 82 to 50%. Western blots for PEPC, PPDK, and NADP-ME indicated that increased protein levels were involved in their induction. The *H. verticillata* NADP-ME polypeptide was larger (90 kD) than the maize C_4 enzyme (62 kD). PEPC and PPDK exhibited up-regulation in the light. Subcellular fractionation of C_4-type leaves showed that PEPC was cytosolic, whereas PPDK and NADP-ME were located in the chloroplasts. The O_2 inhibition of photosynthesis was doubled when C_4-type but not C_3-type leaves were exposed to diethyl oxalacetate, a PEPC inhibitor. The data are consistent with a C_4-cycle concentrating CO_2 in *H. verticillata* chloroplasts and indicate that Kranz anatomy is not obligatory for C_4-type photosynthesis. *H. verticillata* predates modern terrestrial C_4 monocots; therefore, this inducible CO_2-concentrating mechanism may represent an ancient form of C_4 photosynthesis.

Air and water differ considerably in the availability of inorganic C for photosynthesis. Because of the high diffusion resistance of water, the DIC supply rate rather than Rubisco activity can be the major limitation to CO_2 assimilation by submersed aquatic plants (Madsen and Sand-Jensen, 1991). Furthermore, in waters that are densely populated by microphytes or macrophytes, daytime $[CO_2]$ can decline to values far below the K_m for Rubisco and close to the photosynthetic Γ (Van et al., 1976; Talling, 1985; Madsen and Sand-Jensen, 1991). Concomitantly, dissolved $[O_2]$ can increase to more than twice air saturation (Van et al., 1976). Aquatic autotrophs have developed a miscellany of ways to cope with limited $[CO_2]$ and high $[O_2]$. These include various CCMs and the capacity to utilize HCO_3^- for photosynthesis (Bowes and Salvucci, 1989). About 50% of submersed angiosperms can use HCO_3^- in addition to CO_2 for photosynthesis (Madsen and Sand-Jensen, 1991). Such access to HCO_3^- is advantageous, because in many natural waters HCO_3^- constitutes more than 95% of the DIC.

In addition, at least three members of the Hydrocharitaceae show evidence of appreciable C_4-acid metabolism in the light, including high PEPC activity and fixation of radiolabeled C into malate and aspartate (Brown et al., 1974; DeGroote and Kennedy, 1977; Browse et al., 1980; Salvucci and Bowes, 1983). Of the three species *Hydrilla verticillata, Egeria densa,* and *Elodea canadensis,* only in *H. verticillata* has C_4-acid metabolism been shown to be predominantly a light-dependent process closely coupled to photosynthesis (Bowes and Salvucci, 1989). In addition, pulse-chase experiments with *H. verticillata* in the light have demonstrated that the ^{14}C label in C_4 acids can be rapidly chased into sugar phosphates and carbohydrates (Salvucci and Bowes, 1983). Like the use of HCO_3^-, fixation into C_4 acids could be part of a CCM to improve access to limiting [DIC]. Both may be ancient traits among submersed angiosperms, because the Hydrocharitaceae is a submersed monocot family that may extend as far back as 100 million years into the Cretaceous period (Sculthorpe, 1967; Kvaček, 1995).

The C_4-acid cycle in *H. verticillata* is induced in the field or laboratory when the plant is grown under long photoperiods and high temperatures, both of which result in very limiting $[CO_2]$ conditions in the water (Holaday et al., 1983). Both gas-exchange and biochemical data provide evidence that under these low-CO_2 conditions *H. verticillata* leaves shift from C_3 to a type of C_4 photosynthesis (Bowes and Salvucci, 1989). However, an *H. verticillata* leaf is very small (14 mm long × 3 mm wide), with a lamina that is only two cell layers thick, and consequently it lacks the characteristic Kranz anatomy of terrestrial C_4 species (Reiskind et al., 1989). Immunocytochemical gold-labeling and fluorescence studies indicate that PEPC and Rubisco are

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Abbreviations: AT, aminotransferase; CCM, CO_2-concentrating mechanism; DIC, dissolved inorganic carbon; DOA, diethyloxalacetate; Γ, CO_2 compensation point; LN_2, liquid nitrogen; MDH, malate dehydrogenase; NAD-ME and NADP-ME, NAD- and NADP-dependent malic enzyme; PEPC, PEP carboxylase; PPDK, pyruvate orthophosphate dikinase.

present in the cytosol and chloroplast, respectively, of all leaf cells and are not segregated into separate cell types as they are in terrestrial C_4 plants (Reiskind et al., 1989, 1997).

Direct measurements of internal leaf DIC in *H. verticillata* have demonstrated that the C_4-type system is a chloroplastic CCM that overcomes photorespiration (Bowes and Reiskind, 1987; Reiskind et al., 1997). Although *H. verticillata* leaves can utilize HCO_3^- in the medium, this process does not appear to be directly coupled to the operation of the CCM (Reiskind et al., 1997). To date, among submersed autotrophs the *H. verticillata* system is the only substantiated CCM that relies on a form of C_4 photosynthesis. Although there is evidence that a C_4-type CCM operates in the marine macroalga *Udotea flabellum* (Reiskind and Bowes, 1991), its thallus [DIC] has not been measured.

The *H. verticillata* photosynthetic system presents some fascinating problems with respect to its regulation, both in terms of the induction of the C_4-cycle CCM, and its effective operation in the absence of Kranz anatomy. With regard to the induction process, we hypothesize that during the shift from C_3- to C_4-type photosynthesis, increased activities of key C_4-cycle enzymes should correspond to decreases in photorespiration. In this study we examined the role of three key C_4-cycle enzymes in the *H. verticillata* induction process, PEPC, PPDK, and NADP-ME, by determining their activities and steady-state protein levels and whether changes in these biochemical parameters during the induction time coincide with physiological changes in gas-exchange characteristics.

In a facultative CAM plant the shift from C_3 gas exchange to CAM is accompanied by increased activities of C_4-acid-metabolism enzymes (Cushman and Bohnert, 1997). However, *H. verticillata* is not a CAM plant, because its dark CO_2 fixation is only 11% of that in the light, and diel fluctuations in malic acid are small (Holaday et al., 1983; Reiskind et al., 1997). Consequently, unlike CAM plants, in which certain C_4-enzyme activities are highest at night (e.g. PEPC), posttranslational regulation of C_4-cycle enzymes in *H. verticillata* leaves should lead to increased activities during the day. Furthermore, if PEPC is a key component of a C_4-based CCM, its inhibition in the light should result in C_3-like gas-exchange characteristics.

Another crucial consideration in any C_4 photosynthesis scheme is the location of the carboxylating enzymes relative to the decarboxylase, so that exposure of Rubisco to the released CO_2 is maximized, and futile recycling through PEPC is minimized. We have hypothesized that *H. verticillata* utilizes NADP-ME for decarboxylation and that it should be present in the chloroplasts (Bowes and Salvucci, 1989). Here we provide evidence for its intracellular locale, along with other key enzymes in the process.

MATERIALS AND METHODS

Plants of *Hydrilla verticillata* (L.f.) Royle in the C_3-type photosynthetic state were collected from open water in Newnans Lake (Alachua County, FL) or Lake Oklawaha (Putnam County, FL). The plants were thoroughly washed to remove epiphytes. Shoots (8 cm long), containing 75 to 100 leaves (of which more than 90% were fully expanded), were maintained in tap water in 5-L aquaria with a 25°C, 12-h photoperiod and a PPFD of 250 μmol m^{-2} s^{-1}. From this stock, three shoots per tube were placed in 3.5- × 20-cm test tubes containing 80 mL of 5% (v/v) Hoagland solution and incubated for up to 15 d under a 30°C/14-h photoperiod with a PPFD of 250 μmol m^{-2} s^{-1} and a 22°C scotoperiod. The Hoagland solution was changed every other day, and the pH and [DIC] were allowed to fluctuate with the metabolism of the plants. Plants were sampled throughout the incubation period to follow induction of the C_4-type state.

Gas-Exchange Measurements

To measure the Γ, an IR gas analyzer was utilized in a closed system (Van et al., 1976). Shoots were immersed in 100 mL of 5 mM Mes-NaOH and 5% (v/v) Hoagland solution at pH 5.5 in a 200-mL gas-washing bottle with a fritted glass filter through which a gas mixture containing 21% (v/v) O_2 and 50 to 100 μL CO_2 L^{-1} was circulated. The system was then closed, and the plants were allowed to equilibrate to Γ at 30°C with a PPFD of 300 μmol m^{-2} s^{-1}. Measurements were made at pH 5.5 to minimize interference from HCO_3^-.

Photosynthesis rates were measured as net O_2 evolution at 30°C and a PPFD of 300 μmol·m^{-2} s^{-1} using an O_2 electrode system (Hansatech Instruments, King's Lynn, UK). Sprigs (about 1.0 cm long and weighing approximately 50 mg) were immersed in 2 mL of 20 mM Hepes-NaOH, pH 8.0, equilibrated at 21 or 1% (v/v) gas-phase O_2 (equivalent to 246 and 12 μM in solution, respectively), and photosynthesis was initiated by adding $NaHCO_3$ to a final concentration of 1 mM DIC (equivalent to 19 μM free CO_2).

For experiments with the PEPC inhibitor DOA (Sigma), a stock solution containing 3 mM DOA, 0.1 mM EDTA, and 0.5 M KCl was made on the day of use (Bruice and Bruice, 1978). The sprigs were incubated for 4 h at 30°C and a PPFD of 300 μmol m^{-2} s^{-1} in 10 mL of 10 mM Hepes-NaOH, with 100 μM DOA, 2.7 μM EDTA, and 13.4 mM KCl, pH 8.3. As a control, sprigs were incubated in a similar solution that lacked the DOA. Net photosynthetic O_2 evolution rates were then measured as described previously.

Enzyme Activities and Western Analyses

Extracts were prepared from 1.5-cm-long sprigs that were harvested at intervals throughout the induction period. Except where indicated, samples were taken midway into the light period and rapidly frozen in LN_2. Samples of 100 to 250 mg were finely powdered with LN_2 and sand in a mortar and pestle and then homogenized in ice-cold extraction medium. The PEPC extraction medium contained 200 mM Hepes-NaOH, 5 mM DTT, 10 mM $MgCl_2$, and 2% (w/v) PVP-40, pH 7.0 (Hatch and Oliver, 1978). The Rubisco extraction medium contained 50 mM Bicine-HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM DTT, 10 mM isoascorbate, and 2% (w/v) PVP-40, pH 8.0 (Vu et al., 1983). Asp and Ala AT were extracted as described by Hatch and Mau (1973). NADP-ME was extracted with 50 mM Hepes-NaOH, 2 mM $MgCl_2$, and 0.5% (v/v) Triton X-100, pH 8.0

(Hatch and Kagawa, 1974) and then passed through a 9.0-mL Sephadex G-25 column equilibrated at 4°C with 25 mM Hepes-NaOH, pH 8.0. PPDK was extracted in 50 mM Hepes-NaOH, 10 mM DTT, 10 mM $MgCl_2$, 0.2 mM EDTA, 2 mM KH_2PO_4, and 2.5 mM pyruvate, pH 7.4 (Hatch and Slack, 1968).

All enzyme assays were performed at 25°C. Rubisco was assayed radiometrically in both the activated and initial (nonactivated) state (Vu et al., 1983). All other enzymes were assayed spectrophotometrically by following changes in A_{340}. PEPC activity was determined at pH 8.0 in a coupled reaction with MDH, as described by Jiao and Chollet (1988), except that 10 mM $NaHCO_3$ and 5 mM DTT were used. The activities of NADP-ME and NAD-ME were assayed at pH 8.3 and 7.2, respectively (Hatch and Mau, 1977). PPDK activity was assayed in a coupled reaction with PEPC and MDH (Jenkins and Hatch, 1985). Asp and Ala AT were assayed by the method of Hatch and Mau (1973). The procedure of Johnson and Hatch (1970) was used to determine NADPH-MDH activity. Fumarase activity was measured according to the method of Boutry et al. (1984). Total chlorophyll was measured by the method of Arnon (1949).

For western analyses extracts from the *H. verticillata* sprigs harvested at intervals throughout the induction period and from expanded leaves of 5- to 7-week-old maize plants were subjected to SDS-PAGE with 12% (w/v) acrylamide (Laemmli, 1970). The proteins were then blotted onto nitrocellulose membranes, blocked with 5% (w/v) nonfat powdered milk, and probed with polyclonal antibodies raised in rabbits against wheat PEPC (1:1000 dilution), maize PPDK (1:1000 dilution), maize 62-kD NADP-ME (1:1000 dilution), and tobacco Rubisco antiserum (1:250 dilution). With the exception of NADP-ME they were then probed with goat anti-rabbit IgG conjugated to alkaline phosphatase and visualized by a colorimetric detection procedure (Blake et al., 1984). NADP-ME was probed with the above IgG conjugated to biotin and detected with an amplified alkaline phosphatase system (Bio-Rad).

Enzyme Localization Procedures

Leaves (approximately 6.0 g fresh weight) from *H. verticillata* plants with low Γ values were chopped with a razor blade at 4°C in a 1:2 (w/v) homogenizing solution of 50 mM Tris-HCl, 1 mM NaEDTA, 5 mM 2-mercaptoethanol, 10 mM KH_2PO_4, 500 mM Suc, 1% (w/v) BSA, and 0.1% (w/v) PVP-40, pH 7.6. The resultant slurry was squeezed through eight layers of cheesecloth and then by suction filtered through a 10-μm nylon mesh. The filtrate was centrifuged at 15,900*g* for 10 min at 4°C. The supernatant fraction was assayed for enzyme activities. The pellet was resuspended in 10 mM KH_2PO_4, 500 mM sorbitol, and 0.5% (w/v) BSA at pH 7.2, layered onto a stepwise gradient consisting of 2 mL of 50% (v/v) Percoll and 3 mL each of 45, 40, and 30% (v/v) Percoll, and centrifuged at 7,500*g* for 10 min. The chloroplastic, mitochondrial, and pellet fractions were withdrawn and analyzed for enzyme activities.

Unless otherwise stated all data are expressed as the means ± SE of three replicate measurements.

RESULTS

Changes in Photosynthesis Characteristics

H. verticillata plants in the C_3-type photosynthetic state, when incubated under a 30°C, 14-h photoperiod, showed a linear decline ($r^2 = 0.96$) in Γ values from about 60 to 20 μL CO_2 L^{-1} during a 12-d period (Fig. 1). During this time new leaf production was minimal; therefore, the decline was mainly the result of changes in the mature leaves. The Γ values were all measured in solutions equilibrated with 21% gas-phase O_2 to ensure that photorespiratory CO_2 loss could be detected and at pH 5.5 to minimize the HCO_3^- component and its utilization.

To determine the degree to which O_2 inhibited photosynthesis, the net photosynthesis rates of *H. verticillata* were followed over the same 12-d induction period in solutions equilibrated with 21 or 1% gas-phase O_2 (Fig. 1). Concomitant with the decrease in the Γ values, O_2 inhibition also declined in a linear fashion ($r^2 = 0.81$), from 43 to 14%. During this period the O_2 evolution rates measured in 21% O_2-equilibrated solutions doubled from 16 to 32 μmol O_2 g^{-1} fresh weight h^{-1}, whereas those measured at low O_2 increased by only 24%. Thus, the decrease in O_2 inhibition was largely attributable to the doubling in net photosynthesis at 21% O_2.

The in vitro activity of PEPC from terrestrial C_4 plants is completely inhibited by 60 μM DOA (Walker and Edwards, 1990). To investigate its effect on the gas-exchange characteristics of *H. verticillata*, sprigs from C_3- and C_4-type plants were exposed to 100 μM DOA for 4 h. The C_3-type plants in the absence of DOA exhibited substantial O_2 inhibition of photosynthesis (Table I). For these plants DOA did not affect the photosynthesis rates or the degree of O_2 inhibi-

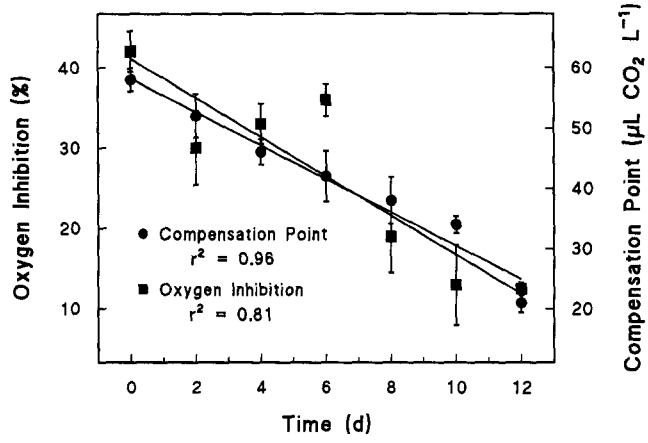

Figure 1. Γ and percentage of O_2 inhibition of photosynthesis of *H. verticillata* leaves as a function of induction time for the plants under a 30°C, 14-h photoperiod. Photosynthetic rates in 21% (v/v) gas-phase O_2 at d 0 and d 12 of the induction period were 16 and 32 μmol O_2 g^{-1} fresh weight h^{-1}, respectively. The Γ and O_2 inhibition data represent the means ± SE of 7 to 11 and 6 measurements, respectively.

Table I. *The effect of DOA on the O_2 inhibition of photosynthesis of C_3- and C_4-type H. verticillata leaves*

Data are the means ± SE of six replicates.

Photosynthesis Type	[DOA]	Photosynthesis Rate		O_2 Inhibition
		21% O_2	1% O_2	
	μM	$\mu mol\ g^{-1}$ fresh wt h^{-1}		%
C_3	0	6.2 ± 2	16 ± 1	61
	100	8.4 ± 1	19 ± 3	56
C_4	0	32 ± 2	39 ± 2	18
	100	21 ± 3	36 ± 3	42

tion. In contrast, DOA treatment of C_4-type plants resulted in a 34% decrease in the photosynthesis rate measured in 21% O_2-equilibrated solution, but the rate measured in 1% O_2 showed little change. Thus, a consequence of the DOA treatment was a greater than 2-fold increase in the percentage of O_2 inhibition of photosynthesis.

Changes in Enzyme Activities and Abundance

Time courses for the induction of the major C_4-cycle enzymes in *H. verticillata* were examined. The activity of PEPC increased rapidly during the first 3 d of induction, increasing from 10 to 120 μmol g^{-1} fresh weight h^{-1}, eventually reaching steady-state values as high as 160 μmol g^{-1} fresh weight h^{-1} (Fig. 2A). Overall, PEPC increased in activity by 16-fold, but the rate of increase did not mirror the gradual decrease in Γ and O_2-inhibition values.

Although PEPC activity increased with induction time, total Rubisco activity did not (Fig. 2A). Thus, the ratio of Rubisco to PEPC activity decreased substantially from 9.0 to 0.2 during a 12-d induction period, although the greatest proportion of this change occurred by d 3. Initial Rubisco activities were also measured to determine whether the activation of this enzyme changed. During the course of the induction period the Rubisco activation state declined from 82 to only 50% because of an approximately 2-fold decrease in initial activity by d 15.

The activities of two other C_4-cycle enzymes, NADP-ME and PPDK, although lower than that of PEPC, also increased substantially during the induction period (Fig. 2B). The approximately 10-fold increase in the activity of both enzymes occurred over a 15-d period, and these time courses were more reminiscent of those associated with the declines in Γ and O_2-inhibition values. To ensure that PPDK activity was indeed being measured in the C_4-type *H. verticillata* leaf extracts, its substrate dependency was examined (Holaday and Bowes, 1980). When pyruvate, P_i, or ATP was omitted from the assay medium the rate was reduced by more than 92%. Likewise, to ensure that NADP-ME and NAD-ME activities were being distinguished, their pH dependency was investigated (Hatch and Mau, 1977). For the NADP-dependent reaction, a decrease in assay pH from 8.3 to 7.2 reduced the activity by 91%. In contrast, NAD-ME exhibited high activity at pH 7.2 but decreased by 98% when the pH was increased to 8.3. Thus, measuring NADP-ME and NAD-ME at pH 8.3 and 7.2, respectively, effectively separated the two activities in the crude extract.

During the induction process Asp and Ala AT activities increased quite rapidly, with all of the increase occurring by d 3 (Fig. 2C). As with PEPC, these activity increases did not correspond with the rate of change in Γ and O_2-inhibition values.

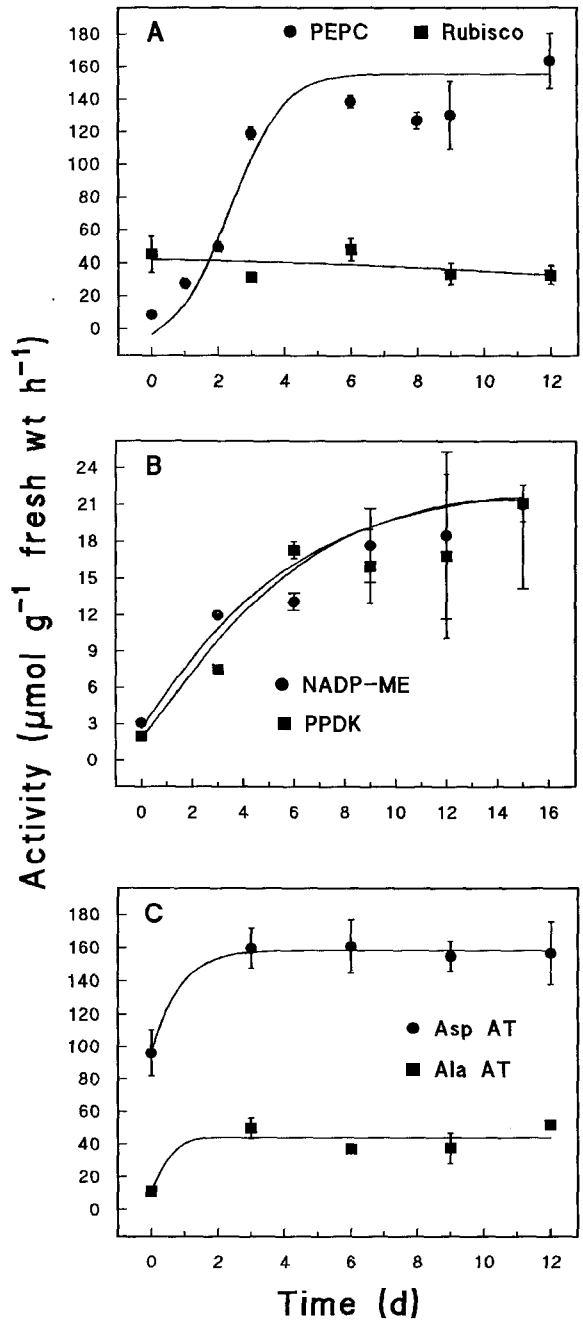

Figure 2. The activities of key enzymes in extracts from *H. verticillata* leaves as a function of induction time for the plants under a 30°C, 14-h photoperiod. A, PEPC and total (activated) Rubisco activities. B, NADP-ME and PPDK activities. C, Asp and Ala AT activities.

Western analyses were undertaken to ascertain whether changes in steady-state protein levels contributed to the increased activities of the three key C_4-cycle enzymes. The data are shown in Figure 3, along with Rubisco and maize controls. An increase in the quantity of *H. verticillata* PEPC polypeptide was evident 48 h after initiation of induction, with further increases up to 120 h thereafter (Fig. 3A). Similarly, PPDK showed an increase during the induction process (Fig. 3A), although it was not as dramatic as with PEPC. In contrast to these two C_4-cycle enzymes, the Rubisco large subunit showed no change (Fig. 3A). The NADP-ME polypeptide also increased during the induction period, but the time scale was longer (Fig. 3B). The *H. verticillata* NADP-ME polypeptide was much larger (approximately 90 kD) than that of the 62-kD maize control.

The activities of the three C_4-cycle enzymes were assayed under saturating substrate and optimum pH conditions after rapidly freezing C_4-type plant leaves in LN_2 at various times during a diel cycle (Fig. 4). There was substantial PEPC activity in the dark, but after the lights went on the enzyme exhibited a gradual increase in activity that peaked after 7 h, being about 50% higher than the dark value (Fig. 4A). The activity then seemed to plateau until the lights went out, after which it declined. In a similar manner, the activity of PPDK approximately doubled, with a gradual increase throughout the light period (Fig. 4B). In contrast to PEPC and PPDK, NADP-ME showed no evidence that its activity varied in a diel manner (Fig. 4B).

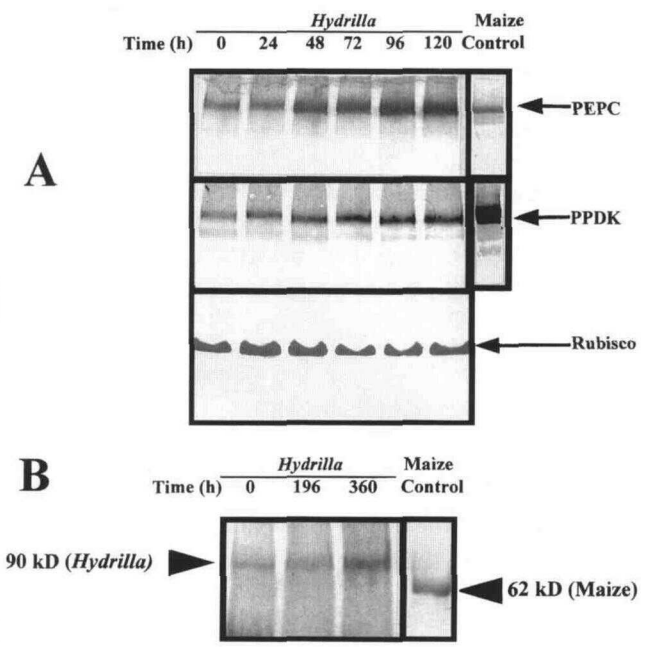

Figure 3. Western analyses of polypeptides corresponding to key enzymes from *H. verticillata* (*Hydrilla*) leaves as a function of induction time for the plants under a 30°C, 14-h photoperiod. A, Top, *H. verticillata* PEPC detected by a wheat anti-PEPC polyclonal antibody; middle, *H. verticillata* PPDK detected by a maize anti-PPDK polyclonal antibody; bottom, *H. verticillata* Rubisco large subunit detected by tobacco anti-Rubisco antisera raised to the native enzyme. B, *H. verticillata* NADP-ME detected by a maize anti-NADP-ME (C_4-type) polyclonal antibody. A maize extract was used as a control.

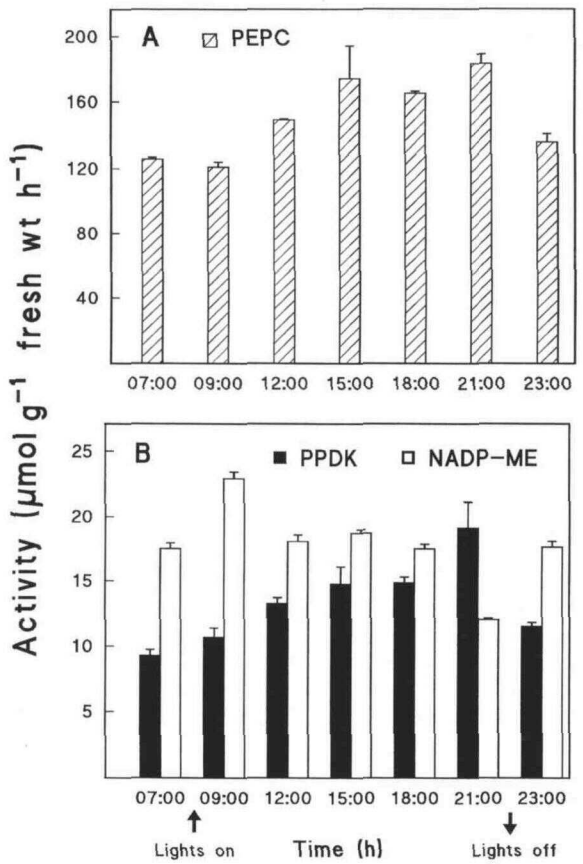

Figure 4. Activities of three C_4-acid enzymes rapidly extracted from C_4-type *H. verticillata* leaves at various times during the light/dark cycle of the plant. The growth chamber lights were turned on at 8 AM, and turned off at 10 PM. A, PEPC activities during the diel cycle. B, PPDK and NADP-ME activities during the diel cycle.

Subcellular Localization of C_4-Cycle Enzymes

The distribution of enzymes potentially involved with C_4-type photosynthesis in *H. verticillata* was ascertained by subcellular fractionation (Table II). The data show that the major portion of the total PEPC activity resided in the supernatant fraction, confirming the cytosolic location of this enzyme in *H. verticillata* (Reiskind et al., 1989). Only 2% of the total activity units was associated with the chloroplastic fraction. In contrast to PEPC, the major location of three other C_4-cycle enzymes, PPDK, NADP-ME, and NADPH-MDH, was the chloroplast, with 82, 91, and 75% of their total activity, respectively, being found in this fraction. However, in the case of NADPH-MDH, approximately 25% of its activity was associated with the supernatant fraction, suggesting that the cytosol was a secondary site for this enzyme. Ninety-five percent of the total NAD-ME activity was found in the mitochondrial fraction, with the remaining 5% associated with the pellet. Similarly, 91% of the activity of the mitochondrial marker enzyme fumarase was associated with the mitochondrial fraction, indicating that the organelles in this fraction were largely intact. Likewise, essentially all of the chlorophyll was in the chloroplast fraction.

Table II. *Distribution of enzymes associated with C_4 photosynthesis in subcellular fractions of C_4-type H. verticillata leaves*

The data include the activity of fumarase, a marker for mitochondria, and the chlorophyll content, a marker for thylakoids. Data are the mean ± SE of six replicates.

Fraction	Enzyme Activity						Chlorophyll
	PEPC	PPDK	NADP-ME	NADPH-MDH	NAD-ME	Fumarase	
	nmol min^{-1}						*μg*
Supernatant	824 ± 30	0	0	156 ± 32	0	0	1 ± 0.2
Chloroplasts	16 ± 9	130 ± 5	58 ± 7	467 ± 157	0	35 ± 9	234 ± 10
Mitochondria	0	18 ± 0.3	2 ± 1	2 ± 1	230 ± 10	538 ± 257	0
Pellet	0	10 ± 0.2	4 ± 2	1 ± 0.6	11 ± 2	20 ± 7	ND[a]

[a] ND, Not determined.

DISCUSSION

The degree to which many submersed freshwater angiosperms photorespire is dependent on the prior growth conditions. Consequently, although terrestrial C_3 and C_4 species have predictable Γ values under defined measurement conditions, this is not the case with submersed species (Bowes and Salvucci, 1989). Under the induction conditions in this study, the Γ of *H. verticillata* declined in a linear manner, and thus the plant displayed a continuum of Γ values from those that were high and like C_3 to low values approaching those of C_4 species. Concomitantly, the O_2 inhibition of photosynthesis decreased substantially. These data are indicative of considerable variation in photorespiratory capacity, which is not unlike the situation with cyanobacteria and microalgae (Badger and Price, 1992), although much less is known about the underlying mechanisms in submersed macrophytes.

The data are also consistent with the induction of a CCM to elevate $[CO_2]$ around Rubisco and thereby reduce the loss of CO_2 via photorespiration. There is direct evidence that a CCM can operate in *H. verticillata*, because measurements of the internal [DIC] in C_4-type leaves show it to be 5-fold higher than that in the bathing medium, generating a chloroplastic free $[CO_2]$ as high as 400 μM (Reiskind et al., 1997). Observed and calculated O_2 inhibition of photosynthesis values as low as zero are consistent with the chloroplasts (rather than the whole cell) being the site where CO_2 is concentrated (Reiskind et al., 1997). By contrast, the [DIC] in C_3-type *H. verticillata* leaves is generally no greater than that in the bathing medium, and O_2-inhibition values are high. From the present study, the linear declines in Γ and O_2-inhibition values suggest that implementation of the *H. verticillata* CCM is gradual, with increasing $[CO_2]$ produced in the chloroplasts as induction progresses.

The CCMs of cyanobacteria and microalgae usually depend on the use of HCO_3^-. Although in *H. verticillata* acidification of the apoplast of abaxial leaf cells enables it to generate CO_2 from HCO_3^- in the medium, this form of HCO_3^- usage is not an obligate component of the CCM (Reiskind et al., 1997). Several lines of evidence support this conclusion: C_3-type leaves without a CCM exhibit pH polarity like the C_4-type, inhibitors of the acidification process do not inhibit the CCM (Reiskind et al., 1997), and, as in this study, low Γ values are attainable in low-pH media containing a minimal $[HCO_3^-]$. The pH polarity of *H. verticillata* leaves demonstrates that aquatic autotrophs may have a CO_2-flux mechanism or CFM, as well as a CCM. The CO_2-flux mechanism improves access to and delivery of external DIC, but, unlike a CCM, it does not increase the internal [DIC] above that of the surrounding medium.

The most probable explanation for the *H. verticillata* CCM is that it is based not on HCO_3^- usage but on a C_4 photosynthetic cycle (Holaday and Bowes, 1980; Salvucci and Bowes, 1981; Holaday et al., 1983; Salvucci and Bowes, 1983). The increase in key C_4-cycle enzyme activities, concomitant with low Γ and O_2-inhibition values, lend credence to the concept that a C_4-type photosynthetic system is induced. The fact that DOA, an inhibitor of PEPC, increased the O_2 inhibition of photosynthesis, but did not decrease HCO_3^- usage (Cooley, 1994), is further evidence for the involvement of a C_4 cycle in the *H. verticillata* CCM. Because DOA did not inhibit the photosynthesis of C_3-type plants or completely abolish it in C_4-type plants, it appears that this compound disrupted the C_4 cycle and the CCM, thereby lowering the chloroplastic $[CO_2]$, but allowed Rubisco to operate on diffusive CO_2.

The marine macroalga *Udotea flabellum* shows a similar partial inhibition of photosynthesis and increased sensitivity to O_2 when its C_4 cycle is disrupted with 3-mercaptopicolinic acid, a PEP carboxykinase inhibitor (Reiskind and Bowes, 1991). The situation in terrestrial C_4 plants is different, because most exhibit more than 90% inhibition of photosynthesis when exposed to 3,3-dichloro-2-(dihydroxyphosphinoylmethyl)propenoate, an inhibitor of PEPC (Jenkins, 1989). This suggests that the ability of terrestrial C_4 plants to function on diffusive CO_2 from the atmosphere is low. Their CO_2 assimilation by Rubisco seems to be almost entirely dependent on the C_4 cycle, perhaps because of diffusion constraints associated with confinement of Rubisco in the bundle sheath.

Although all of the C_4-cycle enzymes investigated in this study increased markedly in activity during the induction period, the time courses for their induction differed. The activity of PEPC showed the most rapid and greatest induction response. These results agree with earlier work showing that PEPC becomes the predominant carboxylating enzyme in C_4-type *H. verticillata* plants (Salvucci and Bowes, 1981). Although both the Γ and O_2-inhibition values declined, they were still relatively high at the end of 6 d, when PEPC had reached almost maximum induction.

This observation indicates that additional components were needed to produce a fully functional CCM. However, increased PEPC activity could recycle photorespiratory and respiratory CO_2. In C_3-type plants of *Egeria densa* and *H. verticillata* , the production of malate in the light, which does not turn over, is consistent with some refixation of CO_2 by PEPC (Browse et al., 1980; Salvucci and Bowes, 1983).

Based on the observation that several days were required for full induction of PEPC activity, it seemed likely that de novo protein synthesis was involved. This is consistent with western analyses, which indicated that PEPC protein levels increased steadily during the first 4 d. In the facultative CAM plant *Mesembryanthemum crystallinum* the activity of PEPC increases over a similar time frame during CAM induction and is also accompanied by increased PEPC protein, as well as by mRNA accumulation (Höfner et al., 1987; Michalowski et al., 1989; Cushman and Bohnert, 1997). In *M. crystallinum* there is a CAM-specific PEPC isogene (*Ppc1*), the expression of which is transcriptionally induced, and an alternate form (*Ppc2*), which is not enhanced (Cushman et al., 1989).

An earlier study of the kinetic properties of PEPC from C_3- and C_4-type leaves provided a hint that different forms might exist in *H. verticillata* (Ascencio and Bowes, 1983). This has now been confirmed, because partial sequences have revealed two isoforms in *H. verticillata* (GenBank accession nos. U65226 and U65227), both of which resemble C_3-type sequences (N.C. Magnin, J.B. Reiskind, and G. Bowes, unpublished data). The latter isoform seems to be much more abundant in C_4-type leaves, but whether this is the one that is induced and functions in C_4-type photosynthesis has yet to be unequivocally resolved (Magnin et al., 1996).

Although an increase in PEPC protein occurred during the induction process, some enhancement of catalytic activity through phosphorylation of preexisting protein cannot be ruled out. The substrate-saturated activity (apparent V_{max}) of PEPC at optimal pH from C_4-type *H. verticillata* leaves exhibited about a 50% increase during the light period. Up-regulation of PEPC activity in the light is characteristic of C_3 and C_4 species, whereas the reverse is true for CAM plants. However, among terrestrial species, covalent modification of PEPC by reversible phosphorylation does not increase the V_{max} at optimal pH but alters the allosteric properties when assayed under near-physiological conditions (Chollet et al., 1996). Therefore, for *H. verticillata* PEPC it is unclear whether the gradual, light-dependent up-regulation of the apparent V_{max} reflected posttranslational modulation or diel oscillation in PEPC protein levels. Further studies are under way to clarify this point.

In the present study total PEPC activity in C_4-type plants was far higher than that needed to support the light- and CO_2-saturated photosynthetic rate and higher than that of most other C_4-cycle enzymes. This suggests that PEPC may not be the major rate-limiting step in *H. verticillata* photosynthesis, although further activity measurements with near-physiological assay conditions are needed to confirm this possibility.

As PEPC activity increased in *H. verticillata*, the ratio of Rubisco to PEPC shifted toward a C_4-like value, and the initial Rubisco activity declined. This caused the activation state to decrease to only 50%. Down-regulation of Rubisco has been reported for a number of species exposed to elevated CO_2, including rice, an emergent aquatic monocot (Bowes, 1993). It is tempting to speculate that similar regulatory phenomena occurred in *H. verticillata* as induction of a CCM increased the $[CO_2]$ around Rubisco.

To operate a C_4 cycle as proposed for *H. verticillata* clearly requires a suite of C_4 enzymes, including a decarboxylase and a means to recycle pyruvate to PEP. The enhancement of Asp and Ala AT activities occurred within the first 3 d; therefore, their induction did not coincide with the change in gas-exchange characteristics. In contrast, the induction rates of NADP-ME and PPDK were more similar to those for C_4-like gas exchange. Their activity increases were also attributable, at least in part, to higher levels of protein. The induction of PPDK in *M. crystallinum* appears to be under regulatory mechanisms different from that of PEPC (Fisslthaler et al., 1995), and this may be the case during C_4-type induction in *H. verticillata*.

The activity of *H. verticillata* PPDK was subject to light regulation on a diel basis. However, the protracted period in the light needed to achieve maximum PPDK activity differs from the rapid dephosphorylation-induced response found in terrestrial C_4 plants and may indicate that diel oscillations in protein levels are involved. In contrast to PEPC and PPDK, the apparent V_{max} of NADP-ME did not change, which makes diel variation in protein synthesis unlikely for this enzyme.

Terrestrial C_4 plants contain at least three isoforms of NADP-ME, two being chloroplastic and one cytosolic (Marshall et al., 1996). Two NADP-ME polypeptides with molecular masses of 62 and 72 kD have been characterized from maize leaves, and the former is associated with C_4 photosynthesis (Maurino et al., 1996). For western analysis of *H. verticillata* NADP-ME, the extract was probed with an antibody raised against the maize "C_4" 62-kD polypeptide. No bands consistent with the 62- or 72-kD subunit isoforms were observed. Instead, a much larger isoform with a subunit of approximately 90 kD was evident, which increased in amount as C_4-type photosynthesis was induced, and thus may represent the C_4-cycle decarboxylase in *H. verticillata*. A 90-kD form of NADP-ME has recently been isolated from maize roots (V.G. Maurino and C.S. Andreo, personal communication), and an antibody to this form also cross-reacted with the extract from C_4-type *H. verticillata* plants (P. Casati, N.C. Magnin, and G. Bowes, unpublished data).

Because *H. verticillata* does not possess the Kranz anatomy and associated intercellular compartmentation of terrestrial C_4 species, we have hypothesized that enzyme segregation is accomplished by organellar compartmentation. *H. verticillata* does not operate as a CAM plant; therefore, a spatial separation of the PEPC and decarboxylase activities would be essential, along with a close association between the decarboxylase and Rubisco to maximize the effectiveness of a CCM. A previous study with *H. verticillata* demonstrated that PEPC and Rubisco are not segre-

gated in different leaf cells but in the cytosol and chloroplasts, respectively (Reiskind et al., 1989). The experiments reported here confirm that the putative decarboxylase NADP-ME and PPDK are confined to the chloroplast, where Rubisco is located. By contrast, the NADP-ME of CAM plants is cytosolic (Edwards and Andreo, 1992).

Another C_4-cycle enzyme, NADPH-MDH, was located predominantly in the chloroplasts of *H. verticillata* leaf cells, but some activity was also detected in the cytosol. If the latter observation is not an artifact, then it differs from the situation in terrestrial plants, where this enzyme is found only in the chloroplast stroma. NAD-ME activity has been detected in *H. verticillata*, and it is higher in C_4-type leaves (Salvucci and Bowes, 1981), but as confirmed here, this is a mitochondrial enzyme and is unlikely to function as the C_4-cycle decarboxylase. If it did, it would release CO_2 into the cytosol, which is inconsistent with data indicating that the chloroplast is the specific CO_2-concentrating site in *H. verticillata* (Reiskind et al., 1997). It would also engender substantial futile refixation by cytosolic PEPC and undermine a CCM. *H. verticillata* leaves have no detectable activity of PEP carboxykinase, a cytosolic decarboxylase found in some terrestrial C_4 and CAM species.

Amphibious sedges in the genus *Eleocharis* also utilize both C_3 and a form of C_4 photosynthesis (Ueno et al., 1988; Uchino et al., 1995). They differ, however, in several important respects from the C_4-type system in *H. verticillata*. The *Eleocharis* system may be a desiccation-induced rather than a low-[CO_2] phenomenon, in that the emergent culms have some C_4-like characteristics, whereas the submersed culms are of the C_3 type. The C_4-cycle decarboxylase is NAD-ME, not NADP-ME as in *H. verticillata*. The most notable difference is that *Eleocharis* depends on Kranz-like anatomy for enzyme compartmentation. However, segregation is not complete, because both mesophyll and bundle-sheath cells contain Rubisco (Ueno, 1996). Incomplete compartmentation of photosynthetic enzymes is a characteristic of terrestrial C_3-C_4 intermediates, such as those in the genus *Flaveria* (Brown and Bouton, 1993). As yet, *H. verticillata* is the only higher plant known to operate a C_4 photosynthetic CCM without Kranz anatomy.

The C_4 system of *H. verticillata* has interesting implications. It demonstrates that a C_4-based CCM does not obligately depend on Kranz compartmentation or access to HCO_3^- at the plasma membrane. Furthermore, the Hydrocharitaceae is an ancient monocot family, and in the case of *Hydrilla*, fossil evidence for this genus has been reported from the upper Eocene of about 40 million years ago (Mai and Walther, 1985). Therefore, *Hydrilla* probably predates modern terrestrial C_4 monocots, which became abundant in the Miocene approximately 7 million years ago (Ehleringer and Monson, 1993). A phylogenetic analysis of *H. verticillata* PEPC sequences is also consistent with the ancient nature of this plant (N.C. Magnin, J.B. Reiskind, and G. Bowes, unpublished data). It is conceivable that the *H. verticillata* system represents an archetypal form of C_4 photosynthesis among angiosperms and that this process occurred in water before its advent on land.

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